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Molecular Studies on Bromovirus Capsid Protein

V. Evidence for the Specificity of Brome Mosaic Virus Encapsidation Using RNA3 Chimera of Brome Mosaic and Cucumber Mosaic Viruses Expressing Heterologous Coat Proteins

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Brome mosaic bromovirus (BMV) and cucumber mosaic cucumovirus (CMV) are structurally and genetically very similar. The specificity of the BMV and CMV coat proteins (CPs) during *in vivo* encapsidation was studied using two RNA3 chimera in which the respective CP genes were exchanged. The replicative competence of each chimera was analyzed in *Nicotiana benthamiana* protoplasts, and their ability to cause infections was examined in two common permissive hosts, *Chenopodium quinoa* and *N. benthamiana.* Each RNA3 chimera replicated to near wild-type (wt) levels and synthesized CPs of expected parental origin when co-inoculated with their respective genomic wt RNAs 1 and 2. However, inoculum containing each chimera was noninfectious in the common permissive hosts tested. Encapsidation assays in *N. benthamiana* protoplasts revealed that CMV CP expressed from chimeric BMV RNA3 was capable of packaging heterologous BMV RNA, however, at a lower efficiency than parental BMV CP. By contrast, BMV CP expressed from chimeric CMV RNA3 was unable to package heterologous CMV RNA. These observations demonstrate that BMV CP, but not CMV CP, exhibits a high degree of specificity during *in vivo* packaging. The reasons for the noninfectious nature of each chimera in the host plants tested and factors likely to affect encapsidation *in vivo* are discussed.

INTRODUCTION

The majority of plant viruses accumulates in the cytoplasm of infected plant cells (Matthews, 1991). To establish a successful infection, a virus needs to protect its genome from degradation, and this is achieved through encapsidation by the virally encoded coat protein (CP). Since viral RNA and CP subunits are localized within the same compartment of the cell (Matthews, 1991), cellular tRNA, mRNAs, or rRNA fragments could potentially be copackaged with viral genomic RNAs. However, specific sequence and/or structure-dependent interactions between CP and viral RNA are envisioned to ensure that the majority of the virions contain exclusively viral RNA (Cuillel *et al.,* 1979).

Heteroencapsidation or genome masking is a mechanism in which the genome of one virus is packaged by the coat protein of another (Matthews, 1991). Such a phenomenon has been observed to occur only between related viruses of the same taxonomic group such as leuteoviruses (Rochow, 1970; Creamer and Falk, 1990; Wen and Lister, 1991), potyviruses (Hobs and McLaughlin, 1990) cucumoviruses (Talianski and Garcia-Arenal, 1995), tobamoviruses (Dawson, 1992), and bromoviruses (Osman *et al.,*1997). Since viral coat proteins play an important role in the interaction of virus particles with its vector (Chen and Francki, 1990; Wen and Lister, 1991), heteroencapsidation could alter the transmission properties of the hybrid virus and result in extended host range. Monocot-adapted brome mosaic bromovirus (BMV) and dicot-adapted cucumber mosaic cucumovirus (CMV) exhibit similarities in physical and biochemical properties (Palukaitis *et al.,* 1992). The genome of the two viruses is partitioned among three RNA components. Viral replication is dependent on two *trans*-acting nonstructural proteins, 1a and 2a, encoded by the monocistronic RNAs 1 and 2 respectively (Ahlquist, 1994; Suzuki *et al.,* 1991; Palukaitis *et al.,* 1992). Unlike BMV, an additional overlapping open reading frame (ORF 2b) is encoded by CMV RNA2, which is expressed through subgenomic RNA (4A), and this gene has been shown to regulate viral movement in tobacco (S. W. Ding *et al.,* 1995). The two gene products encoded by the dicistronic RNA3 of BMV and CMV are dispensable for replication but are required for cell-to-cell spread and systemic infection in plants (Schmitz and Rao, 1996, 1998; Canto *et al.,* 1997). The 5′ proximal ORF of each RNA3 encodes a nonstructural movement protein (MP; Mise *et al.,* 1993; B. Ding *et al.,* 1995). The 3′ proximal ORF of each RNA3 encodes the CP that is translated from a subgenomic RNA4 derived from minus-strand RNA3 (Miller *et al.,* 1986; Boccard and Baulcombe, 1993). The 3′ ends of all four RNAs of each virus display similarities in primary and secondary structures (Palukaitis *et al.,* 1992) and are

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exchangeable between the two viruses (Rao and Grantham, 1994).

The CPs of BMV and CMV are composed of 189 and 218 amino acids, respectively, and assemble into icosahedral particles with T = 3 quasisymmetry (Speir *et al.,* 1995; Wikoff *et al.,* 1997). Recently the three-dimensional structure of CMV was determined by cryoelectron microscopy and X-ray crystallography (Wikoff *et al.,* 1997) and found to be structurally similar to that of another member of the *Bromoviridae,* cowpea chlorotic mottle bromovirus (CCMV). A close similarity was also observed for the quaternary and secondary structures of the CPs of these two viruses (Wikoff *et al.,* 1997). Furthermore, the N-terminal regions of bromo and cucumovirus CPs are basic and contain highly conserved arginine-rich motifs, characteristic of RNA binding proteins (Rao and Grantham, 1996). The CPs of BMV and CMV are multifunctional and participate in various steps during the viral life cycle. In addition to protecting viral genomic RNA, BMV and CMV CPs have been shown to be associated with symptom expression (Shintaku *et al.,* 1992; Suzuki *et al.,* 1995; Rao and Grantham, 1995b, 1996) and cell-to-cell movement (Boccard and Baulcombe, 1993; Taliansky and Garcia-Arenal. 1995; Schmitz and Rao, 1996, 1998; Kaplan *et al.,* 1998; Canto *et al.,* 1997). The above-mentioned similarities between the CPs of bromo and cucumoviruses and the fact that the CMV CP plays a major role in interacting with the insect vector (Chen and Francki, 1990) and the strategy of engineering plants expressing the CMV CP for protection against infection with the same or closely related viruses (Cuozzo *et al.,* 1988), have led us to envision that *trans* encapsidation of BMV genome in CMV CP will have important consequences for the transmission by insect vectors, pathogenesis, and extended host range of BMV. Therefore, this study was undertaken to examine whether the CPs BMV and CMV can package respective heterologous genomes and thus generating biologically active novel hybrid viruses.

RESULTS AND DISCUSSION

We previously demonstrated that the CP genes of monocot-adapted BMV and dicot-adapted CCMV can be freely exchanged between these two bromoviruses and that the chimeric viruses exhibited neutral effects on heterologous hosts (Osman *et al.,* 1997). However, unlike the CPs of BMV and CCMV, which share 70% sequence homology at the amino-acid level (Speir *et al.,* 1995), those of BMV and CMV share only 19% identity (Wikoff *et al.,* 1997), most of which is located in the conserved N-terminal basic arm (Davis and Symons, 1982).

Encapsidation of BMV genomic RNA by CMV CP

To examine whether biologically active CP chimera can be constructed between two distinct members of the *Bromoviridae,* BMV and CMV, CP genes were exchanged using wt cDNA clones of B3 (BMV RNA3) and K3 (CMV-Kin RNA3) (Fig. 1). Cloning manipulations required the introduction of compatible restriction sites at the amino- and carboxyl-termini of the BMV CP (BCP) and CMV-Kin CP (KCP) ORFs. To facilitate replacement of BCP with KCP, restriction sites *Xho*I (compatible with *Sal*I site located at the beginning of BCP ORF; Fig. 1) and *Stu*I (located 12 amino acids upstream to the BCP stop codon; Fig. 1) were created at the beginning and the end of the

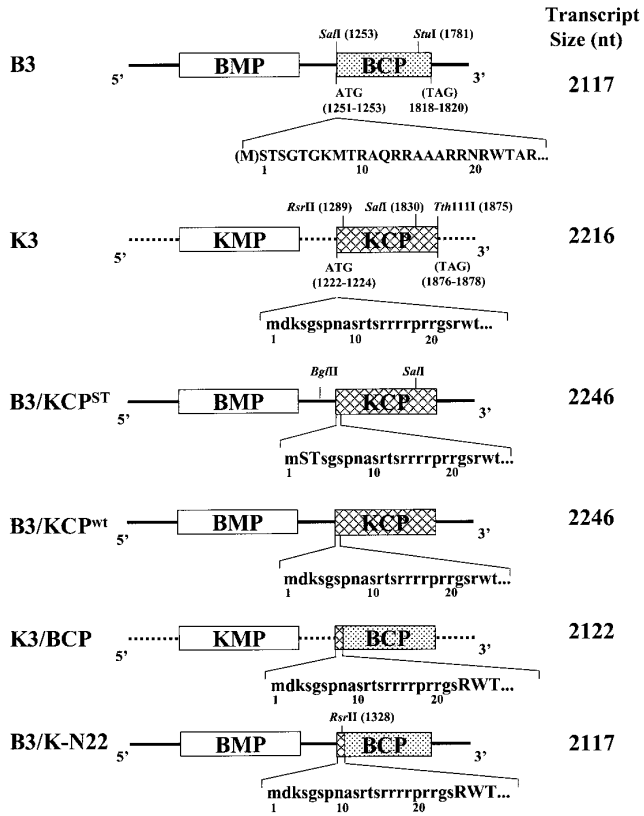

FIG. 1. Characteristics of BMV and CMV CP chimera. The structures of BMV RNA3 (B3) and CMV-Kin RNA3 (K3) are shown. The noncoding sequences of B3 and K3 are represented as solid and broken lines, respectively. The movement proteins of BMV (BMP) and CMV-Kin (KMP) are shown as open boxes. The stippled and crosshatched boxes represent the coat proteins of BMV (BCP) and CMV-Kin (KCP), respectively. The positions of the start (ATG) and stop codons (TAG) of each CP gene are shown. Selected restriction sites used to manipulate the cloning of CP genes between B3 and K3 are shown. The sequences of the first 25 amino acids of the highly conserved N-terminal basic arm of BCP and KCP are shown in uppercase and lower case letters, respectively. In B3/KCPST, creation of a *Xho*I site at the N terminus of KCP resulted in nucleotide changes that retained Ser and Thr of BCP origin (shown in uppercase letters) at positions 2 and 3, respectively. In B3/KCP^{Wt}, Ser^{2} and Thr^{3} were replaced with Asp^{2} and Lys^{3}, respectively to yield KCP with natural N terminus. Sizes of the *in vitro* transcripts expected from wt RNA3 and their are shown at right. *Note*: In BMV, the initiating methionine (shown in parentheses) is removed and the resultant N-terminal serine is acetylated in the mature CP (Moosic *et al.,* 1983) while it is not known whether the initiating methionine is retained in the mature CMV CP. Therefore, the numbering used to denote the N-terminal amino acids of each CP is different.

KCP gene, respectively. RNA transcripts derived from pT7B3/KCPST are designated as B3/KCPST and are 129 nucleotides longer than those of parental wt B3 (Fig. 1). Following the replication of B3/KCPST *in vivo*, the mature CP synthesized from the subgenomic RNA4 of the B3/KCPST chimera will have two amino-acid changes (i.e., dk → ST; Fig. 1) immediately following the KCP initiation codon due to the creation of the *Sal*I site. Unlike the BCP ORF, no compatible restriction site was present at the 5′ end of the KCP ORF sequence, but a unique *Rsr*II was located at position 1289 (22 amino acids downstream of the start codon of KCP; Fig. 1). Since the N-proximal regions of BCP and KCP are highly basic and share extensive amino-acid sequence homology (Rao and Grantham, 1996), an *Rsr*II site was created at an analogous position in the BCP ORF, and the ORF was subcloned into pT7K3 as an *Rsr*II-*Tth*111I fragment (*Tth*111I is located precisely at the end of KCP ORF; Fig. 1). RNA transcripts derived from pT7K3/BCP are designated as K3/BCP and are 94 nucleotides shorter than those of parental wt K3 (Fig. 1). It was anticipated that the CP gene synthesized from the subgenomic RNA4 of K3/BCP will retain the first 22 N-terminal amino acids of the KCP (Fig. 1). When B3/KCPST and K3/BCP were co-inoculated to protoplasts with their respective parental genomic RNAs 1 and 2, each chimera replicated and produced subgenomic RNAs (Fig. 2A) and translated CPs of expected parental origin, as evidenced in Western blots by specific interaction with the respective antiserum (Fig. 2B).

Previous mutational analyses of BMV and CMV CP indicated that this gene product is dispensable for replication (Rao and Hall, 1990; Boccard and Baulcombe, 1993), but is required for cell-to-cell and long distance movement in susceptible host plants (Boccard and Baulcombe, 1993; Schmitz and Rao, 1996; Canto *et al.,* 1997; Rao, 1997). *Chenopodium quinoa* and *Nicotiana benthamiana* are common permissive hosts for BMV and CMV-Kin and display distinguishable symptom phenotypes. In *C. quinoa*, BMV induces chlorotic local lesions followed by systemic infection (Rao and Grantham, 1995b), whereas CMV-Kin induces only local necrotic lesions (Schmitz and Rao, 1998). In *N. benthamiana*, BMV induces symptomless systemic infection (Rao and Grantham, 1995b) whereas CMV-Kin induces a systemic mosaic (Schmitz and Rao, 1998). These distinctive phenotypes are useful markers in analyzing the effect of the heterologous CPs on local and systemic spread. Therefore, inoculum containing *in vitro* transcripts of B1+B2+B3/KCPST (150 μg/ml) was inoculated to four to six *C. quinoa* and *N. benthamiana* plants. A mixture containing all three wt transcripts of BMV served as a positive control and the results are summarized in Table 1. Control plants inoculated with all three wt transcripts displayed the expected characteristic symptoms in each host, and infections were confirmed by Northern and Western blot analysis (Table 1; Fig. 3). However, inoculum containing B3/KCPST failed to induce any visible symptoms in either hosts (Table 1). Even 3 weeks p.i., inoculated plants remained free of visible symptoms. Neither progeny RNA nor CP were detected, in either inoculated or noninoculated systemic leaves of these plants when sensitively probed for viral RNA and CP in Northern and Western blots, respectively (Table 1; Fig. 3). We were unable to induce any infections with B3/KCPST chimera, even when the inoculum dose was increased threefold (Table 1).

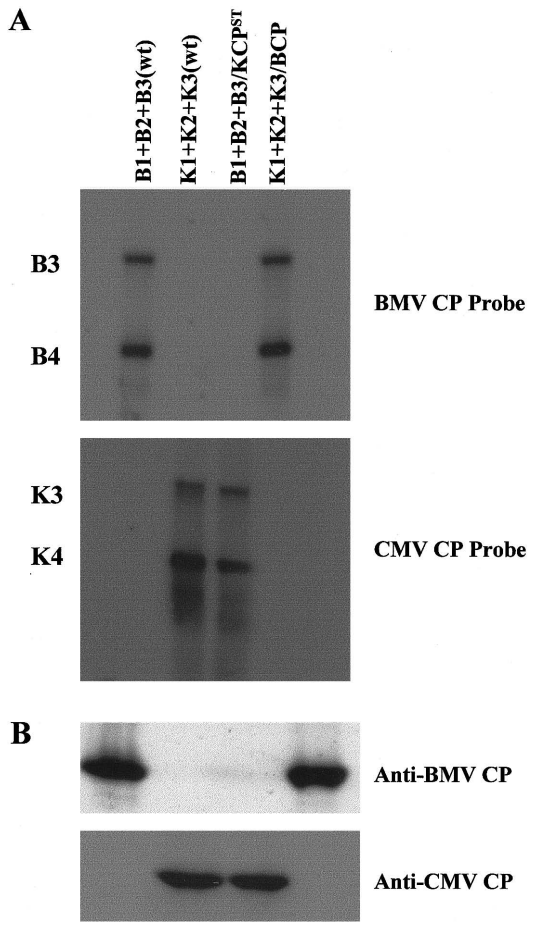

FIG. 2. Progeny analysis of B3 and K3 chimera in *N. benthamiana* protoplasts. (A) Northern analysis of replicative competence of CP chimera in *N. benthamiana* protoplasts. Protoplasts were coinoculated with indicated *in vitro* transcripts. Transfected protoplasts were incubated for 48 h, and RNA was extracted according to Rao *et al.* (1994). RNA was electrophoresed in duplicate gels of 1% agarose after denaturation with glyoxal and transferred to nylon membranes. Blots were hybridized with either ^{32}P-labeled (−) sense RNA probes specific for BMV CP or CMV CP sequences as indicated. The positions of RNA3 and RNA4 of BMV (B3 and B4) and CMV-Kin (K3 and K4) are shown to the left. (B) Western blot analysis of CP accumulation. SDS–PAGE of CP from *N. benthamiana* protoplasts transfected with the indicated inocula. Protoplasts were suspended in SDS–PAGE sample buffer, denatured by boiling for 5 min, and subjected to duplicate gels of 14% polyacrylamide gels. After transferring the proteins to a nitrocellulose membrane, blots were probed with either anti-BMV CP or anti-CMV antibodies (serogroup S) and detected with an enhanced chemiluminescence (ECL) kit (Amersham).

TABLE 1

Infectivity Assays of Parental and Chimeric RNA3 of BMV and CMV

Inoculum[a]	*C. quinoa*			*N. benthamiana*		
	Symptoms (I/S)	WB[b] (I/S)	NB[c] (I/S)	Symptoms (S)	WB[b] (S)	NB[c] (S)
Experiment A (150 μg/ml)						
B1+B2+B3 (wt)	CLL/SM	+/+	+/+	SLI	+	+
B1+B2+B3/KCPST	NI/NI	−/−	−/−	NI	−	−
B1+B2+B3/KCP^{wt}	NI/NI	−/−	−/−	NI	−	−
K1+K2+K3 (wt)	NLL/NI	+/−	+/−	M	+	+
K1+K2+K3/BCP	NI/NI	−/−	−/−	NI	−	−
Experiment B (300 μg/ml)						
B1+B2+B3 (wt)	CLL/SM	+/−	+/−	SLI	+	+
B1+B2+B3/KCPST	NI/NI	−/−	−/−	NI	−	−
B1+B2+B3/KCP^{wt}	NI/NI	−/−	−/−	NI	−	−
K1+K2+K3 (wt)	NLL	+/−	+/−	M	+	+
K1+K2+K3/BCP	NI/NI	−/−	−/−	NI	−	−
Experiment C (450 μg/ml)						
B1+B2+B3 (wt)	CLL/SM	+/+	+/+	SLI	+	+
B1+B2+B3/KCPST	NI/NI	−/−	−/−	NI	−	−
K1+K2+K3 (wt)	NLL	+/−	+/−	M	+	+
K1+K2+K3/BCP	NI/NI	−/−	−/−	NI	−	−

Note. CLL, chlorotic local lesions; SM, systemic mottling; SLI, symptomless infection; NLL, necrotic local lesions; M, mosaic; NI, not infected; wt, wild type.

[a] In each experiment (Experiments A–C), inocula contained a mixture of desired *in vitro* RNA transcripts at the indicated concentration.

[b] CP was detected by Western blot (WB) analysis; I/S, inoculated leaves/systemic leaves; (+) indicates presence of CP; (−) indicates absence of CP.

[c] BMV or CMV RNAs were detected by Northern (NB) analysis; I/S, inoculated leaves/systemic leaves; (+) indicates presence of RNAs; (−) indicates absence of RNAs.

It is well established that CP is indispensable for the cell-to-cell movement of both BMV and CMV (Schmitz and Rao, 1996; Rao, 1997; Canto *et al.,* 1997; Kaplan *et al.,* 1998). However, the form in which CP is required to mediate cell-to-cell movement is distinct for these two viruses. For example, cell-to-cell movement of BMV is dependent on virion formation (Rao and Grantham, 1996; Kasteel *et al.,* 1997), whereas CMV can be transported between cells in a nonvirion form (Kaplan *et al.,* 1998; Schmitz and Rao, 1998). Therefore to examine whether the inability of the inoculum containing B1+B2+B3/KCPST could have been due to the lack of virion formation, we attempted to purify virus particles from $\sim 1.2 \times 10^6$ transfected protoplasts and examined the preparations under an electron microscope. RNA was also extracted from purified virions and analyzed on Northern blots. Although control transfections made with wt inoculum contained numerous virus particles (Fig. 4A), no virions were detected in samples transfected with B3/KCPST. However, transfection of a large sample of protoplasts (4×10^6) with B1+B2+B3/KCPST revealed the presence of virions characteristic of CMV (Fig. 4A). Since CMV cannot assemble into virions without viral RNA being present (Kaper and Geelen, 1971), we surmised that these particles must have packaged BMV RNAs. This assumption was confirmed by probing virion RNA extracted from protoplasts transfected with B1+B2+B3/KCPST in Northern blots with riboprobes specific for CMV CP (Fig. 4B).

The competence of the B3/KCPST chimera to assemble into virions (Fig. 4) taken together with the fact that BMV requires virion formation to mediate cell-to-cell movement (Schmitz and Rao, 1996; Rao, 1997), lead us to expect that inoculation of B3/KCPST to *C. quinoa* should have resulted in an infection. However, this was not the case. We therefore offer two possible explanations for the lack of infection by B3/KCPST: (i) Walker and Pirone (1972) established that $\sim 10^4$–10^7 infectious "virion units" need to be present to manifest a visible local lesion. However, with tripartite viruses, the requirement for more than one particle to be present to form a viral unit and to induce an infection makes the infection process even less efficient than for monopartite viruses. Consequently, alfalfa mosaic virus (AMV), a tripartite virus belonging to the *Bromoviridae*, requires 10^8–10^{10} particles to elicit a local lesion (Matthews, 1991). While *in vitro* transcripts used to inoculate whole plants replicated and synthesized CP to near wt levels (Fig. 2), the same inoculum resulted in inefficient virion assembly, since virus particles were only observed when a large sample of protoplasts was transfected. Therefore, the lack of infection might be attributed to the insufficient number of particles

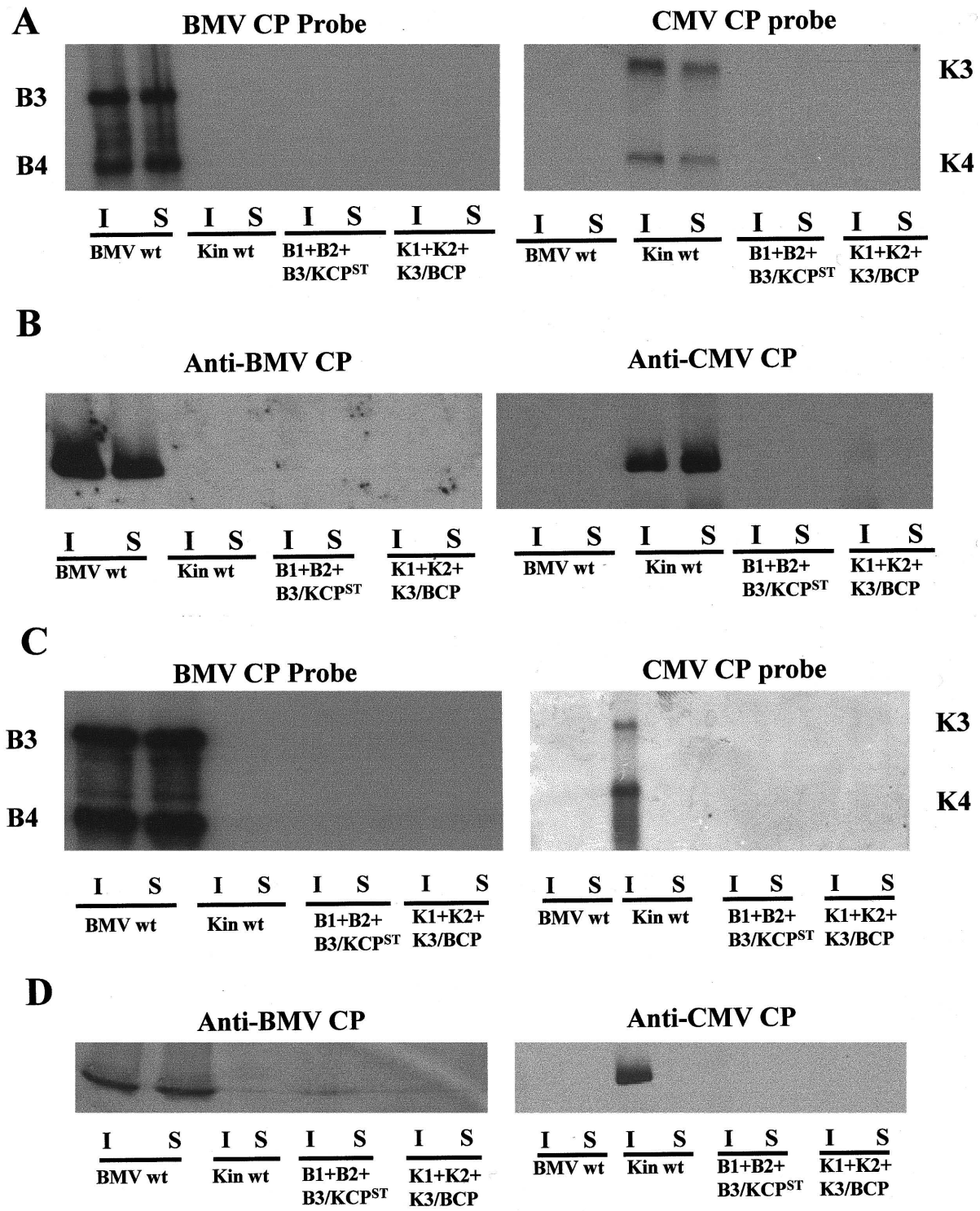

FIG. 3. Progeny analysis from *N. benthamiana* (A and B) and *C. quinoa* (C and D). Northern blot analysis of total RNA recovered from either inoculated (I) or noninoculated systemic leaves (S) of *N. benthamiana* (A) and *C. quinoa* (C) following inoculation with the indicated inoculum. Conditions of Northern blot hybridization and preparation of riboprobes specific for either BCP or KCP are as described under Fig. 2A. Western blot analysis of CP accumulation in either inoculated (I) or noninoculated systemic leaves (S) of *N. benthamiana* (B) and *C. quinoa* (D) with the indicated inocula was done as described under Fig. 2B.

formed to initiate infection. (ii) The most likely factor to affect the infection process was the absence of a compatible interaction between the MP and CP which is obligatory for cell-to-cell movement. It has been shown that in bromoviruses, efficient cell-to-cell movement can occur only when the MP and CP are from the same

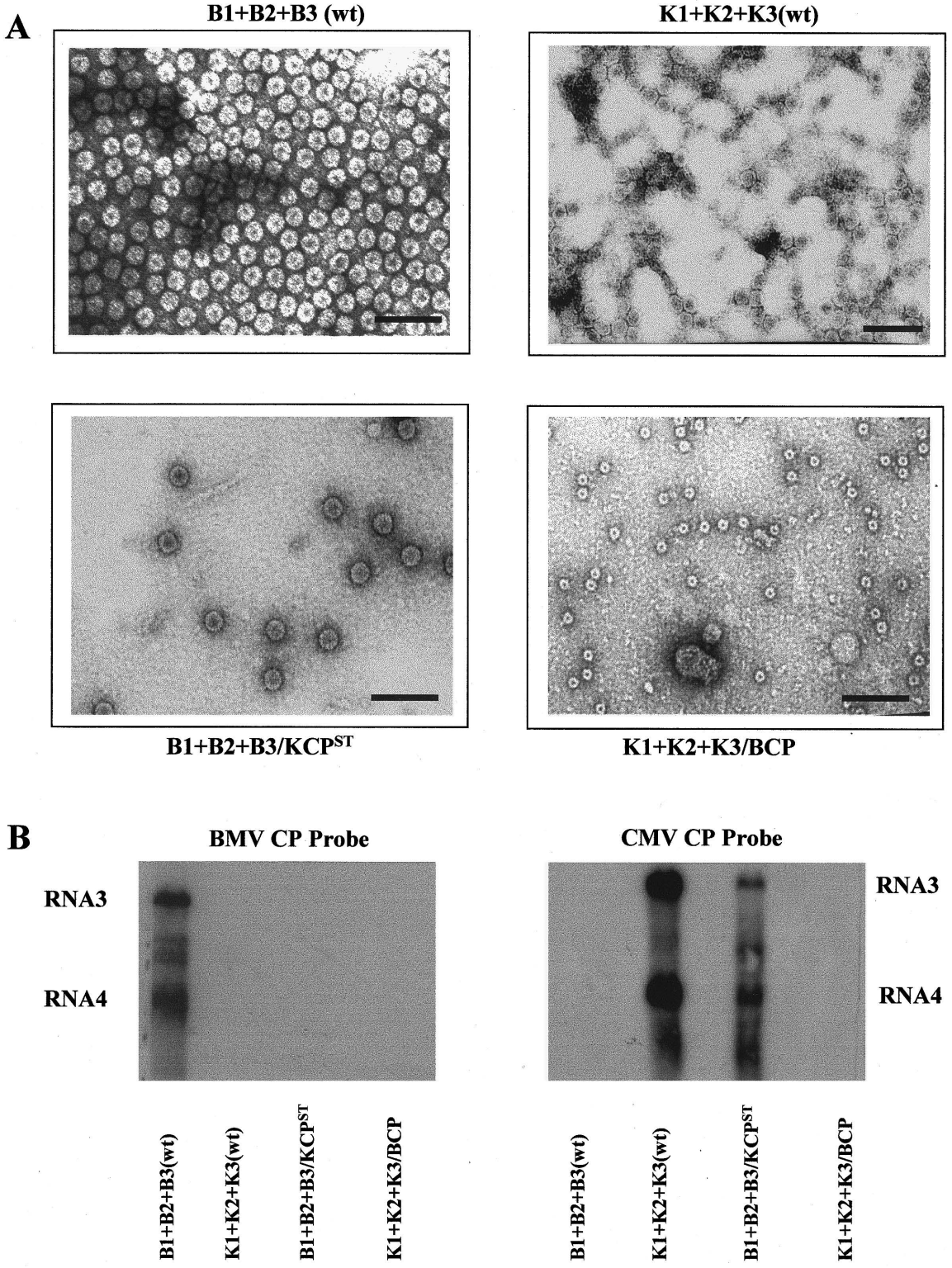

FIG. 4. Electron microscopy of purified virus preparations and RNA analysis. (A) Protoplasts of *N. benthamiana* were transfected with the indicated inoculum, and virions were purified according to the procedure adapted for the parental virus providing the CP gene. Purified virus preparations were negatively stained with uranyl acetate and photographed under a Hitachi electron microscope (Bar = 100 nm). *Note*: Virions shown for B1+B2+B3 (wt) and K1+K2+K3 (wt) were purified from 1×10^6 protoplasts, whereas those for B1+B2+B3/KCPST and K1+K2+K3/BCP were from 4×10^6 protoplasts sample. *Note*: The "virus-like" particles obtained from protoplasts transfected with K1+K2+K3/BCP were identified to be ferritins. (B) Northern analysis of virion RNA of wt and the indicated CP chimera obtained from transfected protoplasts. Virion RNA was subjected to electrophoresis on duplicate gels and each blot was hybridized with the indicated riboprobe. The positions of RNA3 and RNA4 are shown.

parental virus (Mise *et al.,* 1993; Osman *et al.,* 1997). Since wt CMV MP cannot promote cell-to-cell movement of BMV and the chimeric virus failed to induce local lesions in *C. quinoa* (Nagano *et al.,* 1997), the inability of B1+B2+B3/KCPST to induce local lesions may have been due to the lack of proper interaction between BMV MP and CMV CP.

Although defined domains contained within the N-terminal basic arms of BCP and KCP can either be mutated or deleted without having an effect on virion assembly (Rao and Grantham, 1995b, 1996; Schmitz and Rao, 1998), a remote possibility exists that the lack of infectivity and efficient packaging exhibited by B3/KCPST could be due to presence of two N-terminal amino-acid changes (i.e., dk → ST; Fig. 1) in the mature CP synthesized from the subgenomic RNA4 of the B3/KCPST. To verify this possibility, a cDNA clone referred to as pT7B3/KCP^{wt} (Fig. 1) was constructed. Unlike B3/KCPST, inoculation of B1+B2+B3/KCP^{wt} will result in the synthesis of a KCP with natural N terminus. Transfection of B1+B2+B3/KCP^{wt} to protoplasts resulted in efficient synthesis of wt CMV CP and assembled into BMV RNA containing virions indistinguishable from those of B3/KCPST (Fig. 4A). Like B3/KCPST, the inoculum containing B3/KCP^{wt} also failed to induce infection in *C. quinoa*, even when the concentration of the inoculum was increased by twofold (Table 1). Thus it was concluded that the inability of B3/KCPST to infect *C. quinoa* is not due to the presence of two amino-acid changes in the N terminus.

BMV CP exhibits specificity in packaging CMV RNA

To examine encapsidation competence and infectivity of BMV CP, when expressed from CMV, *in vitro* transcripts of K1+K2+K3/BCP were inoculated to *C. quinoa* and *N. benthamiana*. Control inoculations made with wt transcripts of K1+K2+K3 induced expected symptom phenotypes in these hosts (Table 1). As observed with B1+B2+B3/KCPST and B1+B2+B3/KCP^{wt} (Table 1), none of the plants inoculated with K1+K2+K3/BCP displayed any symptoms, and neither viral RNA nor CP was recovered from the inoculated plants (Table 1; Fig. 3). To verify whether BCP synthesized from the subgenomic RNA4 of K3/BCP was competent for assembly into virions containing CMV RNA, an encapsidation assay was carried out in protoplasts. Unlike B3/KCPST or B3/KCP^{wt} transfections, neither virions nor progeny RNA was detected even when a large sample of protoplasts (4×10^6) was transfected with K1+K2+K3/BCP (Table 2; Fig. 4A). However, one of the three independent transfections contained virus-like particles, measuring ~16–18 nm in diameter (Fig. 4A). Northern hybridization with riboprobes specific for the CMV CP sequence failed to detect any virion RNA (Fig. 4B), and immunosorbent electron microscopy using anti-BMV CP revealed that these particles were not assembled from BMV CP (data not shown). These "virus-like" particles were later identified to be ferritin molecules of plant origin (Dr. Desjardins, personal communication). These observations underscore that BMV CP, unlike CMVCP, exhibits a high degree of specificity in packaging viral RNA *in vivo.*

TABLE 2

Encapsidation Assays with Wild-Type and RNA3 Chimera of BMV and CMV

Inoculum[a]	CP type[b]	Northern blots[c]	EM[d]
Experiment A (wt BMV and CMV)			
B1+B2+B3 (wt)	BMV	+	+
K1+K2+K3 (wt)	CMV	+	+
Experiment B (CP chimera)			
B1+B2+B3/KCPST	CMV	+	+
B1+B2+B3/KCP^{wt}	CMV	+	+
K1+K2+K3/BCP	BMV	−	−

[a] Approximately either 1×10^6 (in Experiment A) or 4×10^6 (in Experiment B) protoplasts were transfected with the indicated mixture of *in vitro* transcripts.

[b] CP type was identified by Western analysis of protoplasts transfected with the indicated inoculum.

[c] Virion RNA was isolated and subjected to Northern analysis as described under Materials and Methods. (+) indicates presence of RNA, (−) indicates absence of RNA.

[d] Virions were purified from transfected protoplasts using a procedure adapted for the parental virus providing the CP gene, negatively stained with uranyl acetate and examined under an electron microscope. (+) indicates presence of virions; (−) indicates absence of virions.

In BMV and CMV, the N-terminal basic arms of CP subunits have been implicated in RNA binding (Sgro *et al.,* 1986; Wikoff *et al.,* 1997). Since the first 22 amino acids of the BMV CP derived from the subgenomic RNA4 of K3/BCP are actually of CMV-Kin CP origin (Fig. 1), it is likely that the heterologous N terminus might have failed to interact with BMV RNA. To verify this possibility, chimeric BMV CP was amplified from K3/BCP as a *Sal*I-*Stu*I fragment and subcloned into the genetic background of wt B3, yielding pT7B3/Kin-N22 (Fig. 1). When *C. quinoa* plants were inoculated with B1+B2+B3/Kin-N22, chlorotic local lesions were developed on a time scale similar to that of wt BMV, and the plants later displayed systemic mottling symptoms indistinguishable from those induced by wt BMV. A PCR product, encompassing the chimeric BMV CP gene, was amplified from progeny RNA3 extracted from virions purified from these symptomatic leaves and found to contain an *Rsr*II restriction site, which confirmed the presence of the CMV-Kin N-terminal 22 amino acids (Fig. 1). These observations suggest that N-terminal basic arm is not involved in dictating specificity while interacting with heterologous RNA. This conjecture is further supported by the infectious nature of two BMV CP N-terminal variants in which either the first 25 N-terminal amino acids were replaced

with similar region from TMV or the basic environment was neutralized by substituting the each positively charged amino acid with alanine residues. (Choi and Rao, unpublished data). Taken together, it is obvious that the inability of K1+K2+K3/BCP to induce infection is not due to the presence of the 22 N-terminal amino acids originating from CMV-Kin.

In a previous study, Sacher *et al.* (1988) constructed a BMV RNA3 hybrid harboring the CP gene and the encapsidation origin of a legume strain of TMV (CcTMV). When this hybrid RNA3 was cotransfected to protoplasts with BMV RNAs 1 and 2 (also bearing the CcTMV encapsidation origin), the BMV progeny was packaged into rod-shaped particles. However, Sacher *et al.* (1988) did not construct the reciprocal hybrid to examine whether BMV CP can encapsidate CcTMV RNA. Although previous *in vitro* studies have shown that dissociated bromoviral CP can be reassembled into virions containing RNA from heterologous sources (Hiebert *et al.,* 1968), whether such heteroencapsidation phenomenon in bromoviruses is specific or nonspecific was not know prior to this study. In accordance with Cuillel *et al.* (1979), results of this study clearly demonstrate that BMV CP exhibits specificity during encapsidation *in vivo,* since no virions were recovered from protoplasts transfected with K1+K2+K3/BCP (Fig. 4). These observations parallel to those reported recently for poliovirus (Porter *et al.,* 1998). For example, a poliovirus replicon expressing luciferase (substituted for poliovirus capsid protein P1), when cotransfected with poliovirus type 1, 2, or 3, packaged into type 1, 2, and 3 capsids; by contrast, other picornaviruses such as Coxsackievirus A21 or B3, bovine enterovirus or enterovirus 70 did not result in *trans* encapsidation (Porter *et al.,* 1998). At present the specific packaging signals and CP regions that interact with the genomic RNAs of BMV and CMV are unknown. Although N-terminal basic arms found in the capsid proteins of several plant and animal viruses have been implicated to be the major interacting sites for RNA and located within the capsid shell (Speir *et al.,*1995; Wikof *et al.,* 1997), recent application of matrix-assisted laser desorption/ionization mass spectrometry demonstrated transient exposure of these polypeptides to the viral surface (Bothner *et al.,* 1998). These observations together with the fact that either large deletions in the CPs of BMV and CMV (Rao and Grantham, 1995b, 1996; Schmitz and Rao, 1998) or neutralization of the entire N-terminal basic arm of BMV CP (Choi and Rao, unpublished data) did not abolish infectivity suggested that regions essential for RNA interaction are localized out side the first 25 N-terminal amino acids. Thus the actual role played by the N-terminal basic arm remains to be identified.

Trans encapsidation of the CMV genome by alfalfa mosaic virus (AMV) CP expressed in transgenic plants (Candelier-Harvey and Hull, 1993), raised concerns about the risk for generating novel viral pathogens with altered transmission properties. However, our data demonstrating the inability of the BMV CP to interact with CMV RNA and assemble into particles suggest that the possibility of generating an infectious hybrid virus having the CMV genome encapsidated by BMV CP is low. Additionally, even though the BMV genome was packaged into CMV virions, the reduced rate of virion assembly rendered the hybrid virus noninfectious. Nevertheless, RNA genomes are vulnerable to high mutation rates due to the lack of inherent proof reading ability of RNA-dependent RNA polymerase (Domingo and Holland, 1988); any spontaneous mutations or deletions could influence the interaction between the CP and RNA of two distinct viruses and potentially lead to the generation of new hybrid viral agents.

MATERIALS AND METHODS

cDNA clones

Plasmids pT7B1, pT7B2, and pT7B3 contain wild-type (wt) full-length cDNA clones of BMV RNAs B1, B2, and B3, respectively (Dreher *et al.,* 1989). Plasmids pK1, pK2, and pK3 contain wt full-length cDNA clones of CMV-Kin strain RNAs K1, K2, and K3, respectively (Boccard and Baulcombe, 1992).

Construction of BMV and CMV RNA3 chimera

Construction of pT7B3/KCPST. The genomic maps of wt B3 and K3 are shown in Fig. 1. BMV and CMV-Kin CP genes (designated as BCP and KCP, respectively) were exchanged between cDNA clones of respective RNA3s. To construct a B3 chimera capable of expressing KCP, a DNA product was amplified from pK3 in a PCR reaction (Rao and Grantham, 1996) using a 5′ oligonucleotide primer (dTGTACCTCTCGAGAAAATCT; underlined bases denote a *Xho*I restriction site that is compatible with a *Sal*I site located at the beginning of BMV CP gene sequence; Fig. 1) and a 3′ oligonucleotide primer (dCCGGTAAACACAAGGCCTAAGTCGGG; underlined bases denote a *Stu*I restriction site), and the resulting product was digested with *Xho*I and *Stu*I and subcloned into *Sal*I-*Stu*I-digested pT7B3 to yield, pT7B3/KCPST. The creation of a compatible *Xho*I site in pT7B3/KCPST retained the two N-terminal amino acids from BMV (Ser and Thr located at positions 2 and 3; Fig. 1).

Construction of pT7B3/KCP^{wt}. To modify the Ser^2 and Thr^3 of BCP to Asp^2 and Lys^3 of KCP in clone pT7B3/KCPST, a PCR product was amplified from pT7B3/KCPST using a 5′ oligonucleotide primer (dAAAAAAAGATCTATGTCCTAATTCAGCGTATTAATAATGGACAA ATCTGGATCTCCCAATGCTAGTAGAACC; underline bases denote a *Bgl*II restriction site) and 3′ oligonucleotide primer (dCCGGTAAACACAAGGCCTAAGTCGGG). The resulting product was digested with *Bgl*II and *Sal*I (located at position 1830 of KCP ORF; Fig. 1) and subcloned into

similarly treated pT7B3/KCPST to yield pT7B3/KCP^{wt} (Fig. 1).

Construction of pK3/BCP. To replace KCP with BCP in the pK3 clone, a PCR product encompassing the BCP gene sequence was amplified with a 5′ oligonucleotide primer (dGCTCGCAGAAATCGTTCGGTCCGTAGGGTC; *Rsr*II site is underlined) and a 3′ oligonucleotide primer (dGGGCTCTCCGACCAGGTCCACTACCTATAAA; *Tth*111I site is underlined) and the resulting PCR product was digested with *Rsr*II and *Tth*111I and subcloned into similarly treated pK3 to yield pK3/BCP. The sequence of the subcloned fragments was confirmed by restriction digest mapping and sequencing according to Rao and Grantham (1996).

In vitro transcription and transfection of protoplasts

Prior to *in vitro* transcription, the three genomic wt cDNA clones and the variant RNA3 cDNA clones of BMV were linearized with *Bam*HI (Dreher *et al.,* 1989), those of CMV were linearized with *Bgl*II (Boccard and Baulcombe, 1993). Capped full-length transcripts were synthesized *in vitro* using a MEGAscript T7 kit (Ambion Inc., Austin, TX). For replication and infectivity assays, each of the RNA3 variant transcripts were coinoculated with transcripts of respective parental wt RNAs 1 and 2. Control inoculations contained *in vitro* transcripts of all three parental wt RNAs. *N. benthamiana* protoplasts were isolated using a procedure adapted from the preparation of *C. quinoa* protoplasts (Rao and Grantham, 1996) and transfected using polyethylene glycol (Rao *et al.,* 1994). Progeny RNA was extracted from transfected protoplasts as described previously (Rao *et al.,* 1994; Rao and Grantham, 1995b).

Northern blot analysis and plasmids for riboprobe synthesis

For Northern blot analysis, RNA was denatured with glyoxal, separated on 1% agarose gels and electrophoretically transferred to Nytran membranes (Rao *et al.,* 1994). The blots were hybridized with riboprobes as described by Rao and Grantham (1995a). A T7 RNA transcript of pT73B3SB, constructed by cloning a *Bgl*II-*Sac*I fragment of 261 bases from the pT7B3 CP gene sequence, into pT7/T3–18U (Pharmacia) digested with *Sac*I and *Bam*HI, was used to detect progeny RNA3 and -4 of BMV. A T7 RNA transcript of pT7T3KCP, constructed by cloning a *Hin*dIII–*Eco*RI fragment of 322 bases amplified from the CMV CP gene sequence into similarly treated pT7/T3–18U, was used to detect progeny RNA3 and -4 of CMV.

Western blot analysis

Proteins for Western blot analysis were obtained from either purified virus preparations or transfected protoplasts (2.5×10^5/sample) collected 48 h p.i. Samples were suspended in SDS–PAGE sample buffer [final concentration: 125 mM Tris, pH 6.8,; 10% (w/v) glycerol, 2.5% (w/v) dithiothretol (DTT), 2% SDS, 0.01% bromphenol blue], denatured at 100°C for 5 min, and fractionated on 16% SDS–PAGE according to Laemmli (1970). Fractionated proteins were electrophoretically transferred to a nitrocellulose membrane and detected with either BMV CP antibody (1:1000 dilution) or CMV antibody (1:1000 dilution) using enhanced chemiluminescence (ECL) kit (Amersham).

Packaging assays and electron microscopy

Either barley or *N. benthamiana* protoplasts ($1–4 \times 10^6$/sample) were transfected with *in vitro* transcribed RNA transcripts as described previously (Rao *et al.,* 1994). After 48 h incubation under lights, protoplasts were collected by centrifugation at 400 rpm for 5 min. For samples transfected with either wt BMV or CMV chimera expressing BMV CP, protoplasts were suspended in 0.5 ml of a buffer containing 0.5 M sodium acetate, 0.08 M magnesium acetate, pH 4.5, and 1% β-mercaptoethanol and emulsified with an equal volume of chloroform. The solution was centrifuged for 15 min at 10,000 rpm at 4°C, and the supernatant was centrifuged at 95,000 rpm for 90 min in a Beckman TL 100.2 rotor. A similar procedure was used for protoplasts transfected with either wt CMV or chimeric B3 expressing CMV CP except that samples were suspended in 0.5 ml of a buffer containing 0.5 M sodium citrate, 0.005 M Na-EDTA, pH 6.5, and 0.5% thioglycolic acid, and emulsification with chloroform was omitted. All virus pellets were suspended in buffer containing 0.05 M sodium acetate and 0.008 M magnesium acetate, pH 4.5, and RNA was extracted using SDS and phenol (Rao *et al.,* 1994). For electron microscopy, purified virus preparations were negatively stained with uranyl acetate and observed in an Hitachi H-600 transmission electron microscope.

Biological assays and progeny analysis

N. benthamiana and *C. quinoa* plants were kept in the dark for $\geq$18 h and dusted with Carborundum prior to inoculation. They were mechanically inoculated with 10 μl/leaf of a mixture containing viral RNA transcripts in desired combinations (concentration of 150–600 μg/ml; Rao *et al.,* 1994). Each experiment was repeated at least three times with independently synthesized *in vitro* transcript preparations using four to six plants of *N. benthamiana* and *C. quinoa*. Inoculated plants were kept in the greenhouse at 25°C and observed for symptom expression over a period of 2–3 weeks. Absence of visible symptoms was not considered to be conclusive evidence for the noninfectious nature of the inoculum, and therefore total RNA isolated from asymptomatic leaves was subjected to Northern analysis. Purification of virions from symptomatic leaves and extraction of virion RNA were performed as described above.

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